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Effects of vitamins and inorganic salt in artificial diet on the expression of phytase gene in rBmNPV-Bm expression system

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Abstract:【Aim】Enhancing the yield of silkworm bioreactor and disclosing the relation between the host nutritive condition and the expression of foreign gene in rBmNPV-Bm system.【Methods】The effects of vitamins and inorganic salt in artificial diet for the host insect of baculovirus, silkworm *Bombyx mori*, on the activity of foreign gene products was studied using BmNPV as an expression vector and phytase as a reporter gene.【Results】The activity of phytase in silkworm haemolymph varied remarkably with the amount of added vitamin and inorganic salt. The phytase activity in silkworm haemolymph reached the maximum when the amount of vitamin C was 1% of the dry weight of artificial diet, vitamin B mixture 0.25%, inorganic salts mixture 1% and the amount of vitamin B_6 is 2 mg per 100 g dry matter weight, respectively in the designed test scope.【Conclusions】The expression of phytase gene in BmNPV-Bm expression system could be enhanced efficiently by altering the levels of micronutrients available in the artificial diet for silkworm larvae.

Key words: BmNPV-Bm expression system; vitamin; inorganic salt; phytase gene; expression; enzyme activity

1 INTRODUCTION

The baculovirus expression system has been a vital gene expression tool in the production of numerous recombinant proteins. Two major baculoviruses, *i.e.*, *Autographa californica* nuclear polyhedrosis virus (AcNPV) and *Bombyx mori* nuclear polyhedrosis virus (BmNPV), are widely utilized for the gene expression in insects or their cell lines at the present time. Among them rBmNPV-Bm, using silkworm as the bioreactor, has a far future due mainly to its abundant host insect - silkworm, which can be easily reared on a large scale with the artificial diet throughout the year at low cost. It is very important to further improve the yield of foreign protein in silkworm when the recombinant BmNPV is used as an efficient protein expression vector.

Previous studies revealed that the multiplication of recombinant virus as well as the expression of foreign genes was determined by several factors such as the property of foreign proteins, the integrity of promoter sequence, the presence of fusion protein, and the conditions that influence the infection and multiplication of baculovirus (Lu, 1998). In order to enhance the expression level of foreign genes, two approaches are usually considered: one is to construct the recombinant baculovirus more suitable to the transcription and translation of foreign genes, and the other is performed by modifying the internal and external environments of the host insect. Watanabe and Imanishi (1980) reported that the components of artificial diet had a remarkable effect on the infection of BmNPV and *Bombyx mori* cytoplasmic polyhedrosis virus (BmCPV) in silkworms. Li *et al*. (2000) disclosed that the ingredients of artificial diet significantly influenced the yield of *Spodoptera exigua* nuclear polyhedrosis virus (SeNPV) in *S. exigua*. Lin *et al*. (2000, 2001) observed the marked increase in the expression level of foreign protein in BmNPV-Bm expression system by adding Cu^{2+} and compound nutrition agent in the silkworm diet. Wang *et al*. (2001) revealed the obvious effect of insect juvenile hormone on foreign gene expression. Our former studies showed that the amount of mulberry leaf powder, soybean powder, corn powder and water in artificial diet influenced evidently the expression level of phytase in BmNPV-Bm expression

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system, so the activity of foreign gene product was improved obviously by adjusting the recipe of artificial diet (Shang *et al*., 2003). Using phytase as reporter gene, the effects of the amount of vitamins and inorganic salt on the activity of foreign gene product were further investigated in this study.

2 MATERIALS AND METHODS

2.1 Recombinant virus and silkworms

The *Bombyx mori* recombinant virus harboring the phytase gene was from Dr. Zhang Zhi-Fang, Institute of Biotechnology, Chinese Academy of Agricultural Sciences, Beijing. The silkworm variety 54A × GS was utilized as the host insect for recombinant virus. The test silkworms were reared with artificial diet throughout the larval period and the newly molted silkworm larvae of 5th instar were used as the experiment materials.

2.2 Compositions and preparation of artificial diet

The basic recipe of the artificial diet was listed in Table 1 and the preparation was processed according to the method established in our previous studies (Cui *et al*., 1998). The compositions of vitamin B mixture and inorganic salt mixture were listed in Table 2 and 3, respectively.

Table 1 Basic recipe of artificial diet

Ingredient	Mulberry leaf powder	Bean pulp powder	Corn powder	Vitamin C	Vitamin B mixture	Inorganic salt	Others*
Proportion (%)	30	30	31	1	1	2	5

* Other ingredients included citric acid, gallic acid, sorbic acid, antibiotic, *etc*.

Table 2 Components of vitamin B mixture

Component	Inositol	Choline chloride	Calcium pantothenate	Nicotinic acid	VB_6	Riboflavin	Thiamine hydrochloride	Biotin	Folic acid
Proportion (%)	62.19	31.09	3.11	2.07	0.62	0.41	0.41	0.05	0.05

Table 3 Components of inorganic salt mixture

Component	$K_2HPO_4 \cdot 3H_2O$	$CaCO_3$	KCl	$MgSO_4 \cdot 7H_2O$	$FeSO_4 \cdot 7H_2O$
Proportion (%)	46	26	15	11	2

2.3 Methods

During the first four instars, the silkworm larvae were reared with the basic artificial diet listed in Table 1. The newly molted silkworm larvae of 5th instar were divided into different groups and reared with the diet containing various amounts of vitamin C, vitamin B mixture, VB_6 and inorganic salt. The concentrations of these nutriments were designed according to the common requirements of silkworm and the related reports (Ito *et al*., 1984; Cui *et al*., 2003; Zhang *et al*., 2003), and the corn powder was used as a compensation for the varying levels of vitamin and inorganic salt. Ten larvae were used in each group and all experiments were carried out with three repetitions. At 48 hours of 5 th instar, the larvae were injected subcutaneously with 5 mL of the 1.0×10^5 pfu viral suspension. At 96 hours post infection the haemolymph was collected to measure the phytase activity. The measurement and calculation of phytase activity were carried out according to the method as Wang *et al*. (2001), and the significant difference among various experimental groups was analyzed by statistical analysis method ‘ *t*-test ’.

3 RESULTS

3.1 Effect of vitamin C on the phytase activity in silkworm haemolymph

The effect of vitamin C on the phytase activity was shown in Table 4. The phytase activity achieved the top value when the amount of vitamin C was up to 1% of the dry weight in artificial diet. However, the activity gradually decreased when the additive vitamin C was more than 1%. The phytase activity of the group fed with 4% vitamin C showed a significant decline compared to other groups. These results indicated that vitamin C had important effects on the activity of phytase gene product in baculovirus, and the optimal amount of vitamin C was 1% of dry weight in artificial diet in the scope of the designed test.

Table 4 Effect of vitamin C on the phytase activity in silkworm haemolymph

Additive amount of VC (%)	0	0.5	1.0	2.0	4.0
Phytase activity (U/mL)	125.93 ± 12.32 B	135.43 ± 9.45 B	177.99 ± 17.48 A	122.13 ± 11.09 B	83.37 ± 8.91 C

Notes: The statistical data in the table indicated the mean value ± *SD*, which were all analyzed by *t*-test, and the following capital letter indicated the significance of difference at 1% level in *t*-test. The same below.

3.2 Effect of vitamin B mixture on the phytase activity in silkworm haemolymph

The effect of vitamin B mixture in the diet on the phytase activity was shown in Table 5. The activity of phytase was influenced by the amount of vitamin B mixture and the differences were all above the significance level. When compared to the control (VB 0%), 11.2% increase in the enzyme activity occurred in the silkworm groups reared by the artificial diet containing 0.25% of vitamin B mixture, which indicated the optimal ratio of VB in the scope of the designed test. However, the increase of the concentration of vitamin B mixture beyond this value led to a decrease in the phytase activity.

Table 5 Effect of vitamin B mixture on the phytase activity in silkworm haemolymph

Additive amount of VB (%)	0	0.25	0.50	1.00	2.00
Phytase activity (U/mL)	112.38 ± 13.52 A	125.55 ± 6.35 A	87.17 ± 12.37 B	60.06 ± 8.67 C	53.35 ± 10.12 C

3.3 Effect of vitamin B_6 on the phytase activity in silkworm haemolymph

Vitamin B_6 is the coenzyme of aminotransferases and plays an important role in the metabolism of amino acids in silkworm. The diet was prepared according to the basic recipe in Table 1, but VB_6 was added in different levels here instead of VB mixture. The phytase activity was substantially affected by the additive amount of VB_6 as shown in Table 6. The activity reached as high as 434.60 U/mL when the additive VB_6 was 2 mg per 100 g dry matter. Compared with the control without VB_6, the activity was increased by 4.54-fold. Therefore, it could be assumed that VB_6 was one of the important components for the high-level expression of phytase gene in silkworm, and the optimal amount of VB_6 was lower than that in the basic recipe of artificial diet, which was 6.2 mg/100 g dry matter. Excessive amounts of VB_6 had a reverse effect on the expression of the enzyme.

Table 6 Effect of vitamin B_6 on phytase activity in silkworm haemolymph

Additive amount of VB_6 (mg/100 g)	0	1	2	4	10
Phytase activity (U/mL)	95.67 ± 11.70 D	284.27 ± 19.23 B	434.60 ± 28.84 A	199.53 ± 12.50 C	124.73 ± 8.36 D

3.4 Effect of inorganic salt mixture on the phytase activity in silkworm haemolymph

Based on the basic recipe in Table 1, various amounts of inorganic salt mixture were added in the diet. The phytase activities were examined and the results were presented in Table 7. When compared to the control group, 18.3% increase in phytase activity was found in the group reared by the diet with 1% inorganic salt, which indicated the optimal amount of inorganic salt in the scope of the designed test. However, when the amount of inorganic salt was higher than 1%, it had a negative effect on the phytase activity.

Table 7 Effect of additive amount of inorganic salt mixture in artificial diet on the phytase activity in silkworm haemolymph

Additive amount of inorganic salt (%)	0	1	2	4	8
Phytase activity (U/mL)	105.93 ± 11.05 AB	125.36 ± 8.92 A	92.22 ± 10.58 BC	76.08 ± 10.27 CD	70.44 ± 7.83 D

4 DISCUSSION

Silkworm can be reared by the artificial diet throughout the year and it can be used as a 'biofactory' for the production of useful proteins (Wu *et al*., 2004a, 2004b). In this paper the test silkworms were infected with BmNPV containing phytase gene and the effects of micronutrients in artificial diet on the activity of phytase gene product in silkworm *B*. *mori* were investigated in detail. The results revealed that the amount of vitamin C, vitamin B mixture, vitamin B_6

and inorganic salt all had remarkable effects on the phytase activity. The effect of vitamin B_6 added in the diet was proved to be more efficient than any other components on phytase activity in the host insect. Shang *et al*. (2003) pointed out that the nutritional conditions, which were helpful to the growth and development for silkworms, also enhanced the activity of phytase gene product. Therefore, there was a consistency between the nutrition environment of silkworm growth and the expression efficiency of phytase gene.

Based on the results of this experiment as well as our previous studies (Shang *et al*., 2003), the optimal recipe for the efficient expression of phytase gene in rBmNPV-Bm was obtained as below: mulberry leaf powder 10%, bean pulp powder 35%, corn powder 52.7%, VC 1%, VB mixture 0.25%, inorganic salt 1%, VB_6 2 mg/100 g dry matter, and the additive amount of water was 1.9 times of all the dry matter weight. The recipe needs to perfect further due to the wide span of the additive components in this experiment.

Although vitamin and inorganic salt are not the constitutive material of the silkworm body, nor the direct material of protein synthesis, they are closely related with the metabolism of the host insect of BmNPV and affect the ingestion rate of silkworms as well as the accumulation of nutrients (Ito, 1984; Cui *et al*., 2003; Zhang and Huang, 2003). Xu and Xu (2001) reported that the growth of silk gland was restrained seriously, the protein biosynthesis in haemolymph was abnormal and the transform and utilization of amino acid decreased under the deficient condition of vitamin B_6. In fact, vitamin and inorganic salt have effects on the foreign gene products in many aspects. On one hand, vitamin and inorganic salt could regulate and control the activity of foreign gene products as well as the synthesis of foreign proteins; on the other hand, they might influence directly the foreign proteins, especially the activity of enzymes. The exact mechanism involved in the expression of foreign protein in BmNPV-Bm expression system needs further studies.

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饲料中维生素和无机盐对 rBmNPV-Bm 表达系统植酸酶基因表达活性的影响

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摘要：【目的】为查明宿主的营养对重组家蚕杆状病毒-家蚕表达系统（rBmNPV-Bm）外源基因表达活性的影响，探寻提高家蚕 *Bombyx mori* 生物反应器产率的新途径。**【方法】**以家蚕 BmNPV 病毒为表达载体，以人工饲料饲养的 5 龄家蚕为宿主，以植酸酶基因为报告基因，探讨了家蚕人工饲料中维生素和无机盐对外源基因表达产物活性的影响。**【结果】**家蚕血淋巴中外源植酸酶的活性随着饲料中维生素和无机盐添加量的不同而发生显著变化。在本试验设区范围内，当维生素 C 的含量为饲料干物质量的 1%，B 族维生素混合物为 0.25%，无机盐混合物为 1%，维生素 B_6 为 100 g 饲料干物中含 2 mg 时，血淋巴中植酸酶活性达到最大值。**【结论】**通过改变家蚕饲料中微量营养成分的含量是提高 rBmNPV-Bm 表达系统外源基因表达活性的有效途径。

关键词：BmNPV-Bm 表达系统；维生素；无机盐；植酸酶基因；表达；酶活性

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